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Global response of *Acidithiobacillus ferrooxidans* ATCC 53993 to high concentrations of copper: A quantitative proteomics approach

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ABSTRACT

Acidithiobacillus ferrooxidans is used in industrial bioleaching of minerals to extract valuable metals. *A. ferrooxidans* strain ATCC 53993 is much more resistant to copper than other strains of this microorganism and it has been proposed that genes present in an exclusive genomic island (GI) of this strain would contribute to its extreme copper tolerance. ICPL (isotope-coded protein labeling) quantitative proteomics was used to study in detail the response of this bacterium to copper. A high overexpression of RND efflux systems and CusF copper chaperones, both present in the genome and the GI of strain ATCC 53993 was found. Also, changes in the levels of the respiratory system proteins such as AcoP and Rus copper binding proteins and several proteins with other predicted functions suggest that numerous metabolic changes are apparently involved in controlling the effects of the toxic metal on this acidophile.

Significance: Using quantitative proteomics we overview the adaptation mechanisms that biomining acidophiles use to stand their harsh environment. The overexpression of several genes present in an exclusive genomic island strongly suggests the importance of the proteins coded in this DNA region in the high tolerance of *A. ferrooxidans* ATCC 53993 to metals.

1. Introduction

The biomining acidophilic bacterium *Acidithiobacillus ferrooxidans* can grow at the high copper concentrations (>100 mM copper sulfate) [1,2] that are usually present in biomining processes [2–4]. Current knowledge indicates that key elements involved in *A. ferrooxidans* copper-resistance are a broad repertoire of known copper-resistance determinants and their duplication, the presence of novel copper-chaperones, an abundant reserve of inorganic polyphosphate (polyP) used in a polyP-based copper-resistance system and a defensive response to oxidative stress [5–7]. *A. ferrooxidans* ATCC 23270 can survive high copper concentrations by having in its genome at least ten genes that are possibly related to copper homeostasis, such as those coding for CopA efflux ATPases, Cus efflux systems and copper chaperones [8]. *A. ferrooxidans* ATCC 53993 showed a much higher resistance to $CuSO_4$ (>100 mM) than that of strain ATCC 23270 (<25 mM) [1]. Strain ATCC 53993 genome contains the same copper resistance determinants than strain ATCC 23270. The copper resistance determinants shared by the two strains are: three efflux ATPases $CopA1_{Af}$, $CopA2_{Af}$ and $CopB_{Af}$; an RND system: $CusCBA1_{Af}$ and three copper binding chaperones: $CusF1_{Af}$, $CusF2_{Af}$ and $CopC_{Af}$. Strain ATCC 53993 on the other hand has a 160-kb genomic island (GI) in its genome, which is absent in ATCC 23270. This GI contains, among other genes, the following exclusive coper resistance determinants: the efflux ATPase $CopA3_{Af}$, two RND systems $CusCBA2_{Af}$ and $CusCBA3_{Af}$ and the two copper chaperones $CusF3_{Af}$ and $CusF4_{Af}$.

Copper resistance in strain ATCC 53993 could be explained in part by the presence of the additional copper-resistance genes present in its GI. Some of these genes are transcriptionally overexpressed when *A. ferrooxidans* ATCC 53993 is grown in the presence of copper and the coded proteins are functional when expressed in copper-sensitive *Escherichia coli* mutants [1,4].

Due to the current lack of appropriate and reproducible genetic tools in *A. ferrooxidans* it is not possible to study in more detail its copper resistance mechanism. Therefore, a global quantitative proteomic approach was used in the present work to characterize the response of *A. ferrooxidans* strain ATCC 53993 to a high concentration of copper and to further search for new possible copper resistance determinants in this microorganism. In addition to changes in the levels of several proteins in the genome of this strain, several proteins coded only in its GI were highly up-regulated both transcriptionally and translationally when the microorganisms were grown in the presence of copper. Some of these macromolecules apparently have a key role in the adaptation of the microorganisms to their extreme environment, providing it with a much higher copper resistance compared with those strains lacking this genomic element.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

A. ferrooxidans strain ATCC 53993 was grown at 30 °C in liquid 9 K medium containing ferrous sulfate (33.33 g/L) with an initial pH of 1.45 as previously described [9] and in the absence or presence of $CuSO_4$. Initially, cells were adapted to grow in 5 mM copper (a concentration that does not affect its growth) until they reached the stationary phase of growth. These cells were subcultured in the presence of copper by successively growing and transferring them to different increasing $CuSO_4$ concentrations until they were adapted to 40 mM copper.

Once 40 mM concentration was reached, triplicate separate cultures were done. After the cells attained the late exponential growth phase they were collected for their immediate use in different experiments. Bacterial growth was determined by measuring the increase in cell numbers by using an Olympus BX50 optical microscope and a Petroff–Hausser counting chamber.

2.2. Total protein extracts preparation for ICPL analysis

A. ferrooxidans cells grown in the absence or presence of 40 mM $CuSO_4$ were harvested by centrifugation (10,000 × *g* for 2 min). The cell pellets were washed three times with sulfuric acid solution (pH 1.5) by resuspension followed by centrifugation at the same speed and time already mentioned.

Cells were then resuspended in sonication buffer (50 mM Tris–HCl pH 8, 1 mM EDTA) containing PMSF as protease inhibitor (100 μg/mL) and were disrupted by sonic oscillation during 60 min on ice by using successive 15 s pulses. Finally, the lysate was centrifuged at 10,000 × *g* for 10 min to remove unbroken cells and cell debris and total protein concentrations in the cell-free extract was determined as done before [10].

2.3. Sample preparation for ICPL determinations

Samples of total protein extracts from three biological replicates (different independent cultures) were mixed using fifty micrograms of each one to obtain a triplicate representative sample of each experimental condition with a total of 150 μg of protein in each case. These mixtures were lyophilized for 48 h at −40 °C. Finally, the dried samples were stored at −20 °C until their isotope-coded protein label (ICPL) labeling as described before [10].

2.4. Protein digestion and ICPL-labeling

The ICPL-reagent protocol was optimized for labeling of 100 μg of each individual sample per experiment. Thus, 100 μg of total protein extracts was individually dissolved in 8 M urea 25 mM ammonium bicarbonate, reduced and alkylated with iodoacetamide. Further details are as previously described [10].

2.5. 2D-nano LC ESI-MS/MS analysis

ICPL-labeled combined samples (200 μg per experiment) were dissolved in 100 μL of 10 mM NH_4OH in water, pH 9.5 and fractionated in a wide-pH range 5 μm particle size, 100 × 2.1 mm reversed phase XBridge column (Waters) using a Knauer Smartline HPLC system. Details for gradient elution, flow rate, injection volumes, number of HPCL fractions and second dimension of the 2D-nano LC ESI-MS/MS analysis were as previously reported [10].

2.6. Protein identification and quantitative analyses

MS and MS/MS data obtained for individual HPLC fractions were merged using the Analysis Combiner tool and subsequently processed as a single experiment using DataAnalysis 3.4 (Bruker Daltonics, Bremen, Germany). In most cases, an accuracy of ±0.1–0.2 Da was found both for MS and MS/MS spectra. For protein identification, MS/MS spectra (in the form of Mascot generic files) were searched against the *A. ferrooxidans* ATCC 53993 UniprotKB forward-reversed database (http://www.uniprot.org) containing 2747 entries and their corresponding reversed sequences. Sequence reversal was done using the program DBToolkit v4.1.5. Database searches were done using a licensed version of Mascot v.2.2.04 (www.matrixscience.com; Matrix Science, London, UK). Search parameters were as previously described [10]. FDR ≤ 5% for peptide identification were manually assessed as follows: after database searching, peptide matches were ranked according to their Mascot scores. This list contains peptide sequences matching either forward or reversed database sequences. Then, a subset containing 5% of peptides matching the reversed sequences was extracted.

Qualitative and quantitative analyses were performed by WARP-LC 1.1 (Bruker Daltonics, Bremen, Germany). After peptide identification, the software calculates the extracted ion chromatogram for the putative ICPL-labeled pair according to: (a) the mass shift defined by the labeling reagent, (b) a mass tolerance of 0.5 Da, and (c) a retention time tolerance of 40 s. Relative ratios between light and heavy ICPL-labeled peptides were calculated based on the intensity signals of their corresponding monoisotopic peaks, and according to these individual peptide ratios the software calculates the protein ratio. Protein quantification values based in single (unique) peptides were manually evaluated. Ratios were $\log_2$-transformed and normalized by subtracting the median value.

Only proteins showing $\log_2$ (Cu 40 m/Cu 0 mM) values $R \geq 0.5$ or $R \leq -0.5$ were considered as up- or down-regulated. Protein ratios in the $-0.5 \leq R \leq 0.5$ range ($R = \log_2$ (Cu 40 m/Cu 0 mM)) were considered non-relevant in the context of the biological model studied and their change probably due to artifactual variation (see figure below) associated to the experimental set up used in this case. In addition, to support the consistency of our proteomic data a small and selected subset of proteins considered up or down regulated using this proteomic approach was chosen for further validation using a PCR-based approach.

Proteins changing their levels in the presence of copper were classified according to their functional categories by using the COG (Cluster of Orthologous Groups) database. Those proteins whose ORFs had no associated COGs were classified by using as before [10] the bioinformatics tool COGnitor that allows to assign a COG based on the protein sequence (http://www.ncbi.nlm.nih.gov/COG/old/xognitora.htlm).

2.7. Extraction of total RNA from A. ferrooxidans and cDNA synthesis

To determine the effect of copper on the expression of some genes of interest, cells were adapted in batch cultures to grow in the absence or presence of 40 mM $CuSO_4$ until the late exponential growth phase was reached. At this time, total RNA was extracted standardly from each culture condition by lysing the cells as previously reported [11], except that TRIzol (Invitrogen) was used for the extraction [1,8]. Between three to five biological replicas were used for each experimental condition. Any remaining DNA was eliminated from the RNA preparations by the addition of 4 U of TURBO DNA-*free* DNase (Ambion) following the manufacturer's instructions. For cDNA synthesis, 0.8 μg of total RNA was reverse transcribed for 1 h at 42 °C using ImProm-II (Promega) reverse transcription system, 0.5 μg of random hexamers (Promega) and 3 mM $MgCl_2$ [8].

2.8. Primer design, real-time PCR and cloning of A. ferrooxidans genes

Primers for qRT-PCR were designed using the Primer3 software [12]. After separating PCR products by electrophoresis in a 1% agarose gel (0.5× Tris–acetate–EDTA pH 8.0 buffer), no cross-amplification or non-specific bands were detected. Copper-resistance related gene expression was analyzed by qRT-PCR with the Corbett Rotor Gene 6000

system as described previously [10]. For transcriptional analysis of the different genes studied, a relative quantification method was used which is based in the ratio between a study sample (presence of copper) versus a control sample (no copper) transcripts [13]. 16S rRNA$_{Af}$ was selected as a reference gene since its expression was found to be the most stable under our experimental conditions.

3. Results and discussion

As seen in Fig. 1, metabolism was the functional category with the greatest number of changes in cells grown in the presence of 40 mM copper (28 proteins or 57.2% of the total proteins). Within this category, 28.5% of the proteins (8 out of 28) corresponded to inorganic ion transport and metabolism. This group of proteins is important, since most of the copper resistance mechanisms from other bacteria involve proteins classified in this category. The group of Energy production and conversion also showed substantial changes within the Metabolism category (Fig. 1). Cellular processes and signaling was the second functional category most affected by the presence of copper (24.5%). Within this category, 50% of the changes were in post-translational modification, protein turnover, and chaperones. Information storage and processing showed only 2% of the changes. The remaining proteins (16.3%) were grouped as poorly characterized or with no homologues in databases. These are proteins with unknown functions that may be exclusive to *A. ferrooxidans*.

The ICPL proteomic analysis showed that the proportion of changes of proteins in the different functional categories seen in Fig. 1 for *A. ferrooxidans* ATCC 53993 at 40 mM copper are in general similar to those previously reported for *A. ferrooxidans* ATCC 23270 at the same metal concentration [10]. The proteomic analysis of *A. ferrooxidans* ATCC 53993 identified 523 proteins of which 49 (9.36%) changed their levels significantly compared to control cells grown in the absence of copper. In the group of proteins changing, 33 were up-regulated and 16 down-regulated (Fig. 1). Previous results with the same proteomic approach identified 594 proteins in *A. ferrooxidans* ATCC 23270 of which 129 (21.7%) changed their levels significantly in the presence of copper compared to the control cells [10]. This clearly suggests that the presence of 40 mM copper affects more strain ATCC 23270 than ATCC 53993.

Next, the possible identities and putative functions of several of the differentially synthesized proteins from *A. ferrooxidans* ATCC 53993 are described grouped by their functional categories with predictions of their possible role in copper resistance. Obviously, the majority of these suggestions will require future experimental demonstrations.

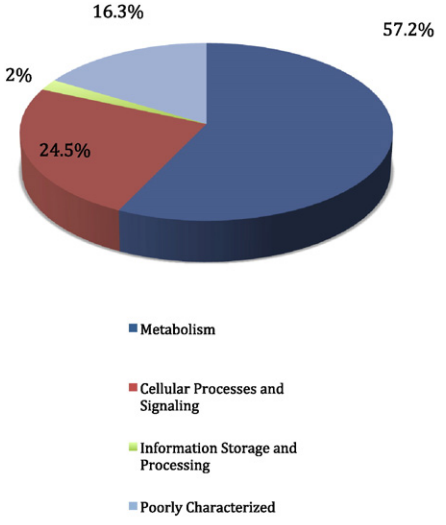
3.1. Inorganic ion transport and metabolism

Table 1 shows highly increased levels of five components of possible RND type efflux systems (CusC1$_{Af}$, CusC2$_{Af}$, CusB3$_{Af}$, CusB1$_{Af}$ and CusA2$_{Af}$) when *A. ferrooxidans* ATCC 53993 was exposed to 40 mM copper. These results were confirmed at the transcriptional level by using qRT-PCR for CusA2 and CusA3 as seen in Fig. 2A. Interestingly, one of these three RND systems (CusCBA3$_{Af}$) is coded in the exclusive GI present in this strain and all of them showed high levels (3 to 4-fold) in cells grown in the presence of 40 mM copper. These results together with those previously reported [1,8,10] strongly support the importance of these RND type of transporters in copper resistance in *A. ferrooxidans*. Furthermore, they might explain in part the much higher copper resistance of *A. ferrooxidans* ATCC 53993 compared to that of *A. ferrooxidans* ATCC 23270 since the former one has three probably functional copies of Cus systems (one in its GI and two in the rest of the genome) compared to only one functional gene of the two present in ATCC 23270, since one of these is interrupted by a transposon [1].

E. coli CusF is a periplasmic protein that forms part of the CusCFBA system and mediates the transfer of Cu to the CusCBA efflux complex to facilitate periplasmic detoxification [14–16]. In addition, it has been recently reported that the copper taken to the periplasmic space by the inner membrane ATPase CopA would be directly transfered to CusF when bound to the periplasmic domain of CopA [17].

A. ferrooxidans ATCC 53993 contains four CusF copper chaperones, two coded only in its GI (CusF3$_{Af}$ and CusF4$_{Af}$) and two on the rest of its genome (CusF1$_{Af}$ and CusF2$_{Af}$). These last two chaperone genes are also present in strain ATCC 23270 [8]. The genetic organization of some of these CusF chaperones differs from that reported in *E. coli*, where the four genes of the CusCFBA are part of an operon [15].

In the Cellular process and signaling category, subcategory Post-translational modification, protein turnover, and chaperones there was a high increase in the levels of the copper chaperones CusF2$_{Af}$ (Lferr_2057) and CusF3$_{Af}$ (Lferr_0174) (Table 1). Both CusF2$_{Af}$ and CusF3$_{Af}$ chaperones were among the three proteins with the highest levels of fold change (ca. 6 fold) at 40 mM copper. Furthermore, at this metal concentration the transcriptional expression of these chaperones as determined by qRT-PCR also increased around 6-fold (Fig. 2B), confirming the proteomic results seen in Table 1. These results suggest



Functional category	N° prot	prot (+)	prot (-)	%
Metabolism				
Inorganic ion transport and metabolism	8	6	2	16.3
Amino acid transport and metabolism	2	2	0	4.1
Carbohydrate transport and metabolism	5	3	2	10.2
Lipid transport and metabolism	2	1	1	4.1
Energy production and conversion	11	8	3	22.5
Cellular processes and signaling				
Post-translational modification, protein turnover, and chaperones	6	6	0	12.3
Cell wall/membrane/envelope biogenesis	5	2	3	10.2
Signal transduction mechanisms	1	1	0	2.0
Information storage and processing				
Translation, ribosomal structure and biogenesis	1	1	0	2.0
Poorly characterized				
General function prediction only	1	1	1	2.0
Function unknown	7	2	4	14.3
TOTAL	**49**	**33**	**16**	**100**

Fig. 1. Functional categories and numbers of *A. ferrooxidans* proteins changing their synthesis levels in cells grown in the presence of 40 mM $CuSO_4$. The pie chart refers only to the proteins listed in the accompanying table, where (+) indicates upregulated and (−) downregulated proteins.

Table 1
Up-Regulation of proteins in *A. ferrooxidans* ATCC 53993 grown in ferrous iron and in the presence of 40 mM $CuSO_4$.

Accession number	ORF	Function/similarity	Score*	Coverage (%)	Peptide number	Fold change (Cu 40/0 mM)
Metabolism						
Inorganic ion transport and metabolism						
B5EM41	2062	Outer membrane efflux protein (CusC2)	73	12	5	4.06
B5EJG7	1619	Outer membrane efflux protein (CusC1)	83	8	4	3.98
B5EK70	171	Efflux transporter, RND family, MFP subunit (CusB3)	150	15	6	3.73
B5EJG6	1618	Efflux transporter, RND family, MFP subunit (CusB1)	52	9	3	3.27
B5EM39	2060	Heavy metal efflux pump, CzcA family (CusA2)	110	5	6	2.99
B5ELI0	1945	TonB-dependent receptor	47	2	6	2.57
Amino acid transport and metabolism						
B5EP37	878	Glutamate synthase (Ferredoxin)	186	6	11	2.09
B5ELM5	1992	Dihydrodipicolinate synthase (DapA)	99	17	6	1.96
Carbohydrate transport and metabolism						
B5EPB6	2462	ROK family protein	79	9	2	12.02
B5ENX7	818	Inositol-phosphate phosphatase	95	6	2	4.19
B5EN05	684	2,3-bisphosphoglycerate phosphor glycerate mutase 1 (gpmI1)	57	4	2	2.05
Lipid transport and metabolism						
B5EMF7	582	Enoyl- [acyl-carrier-protein] reductase [NADH]	394	26	13	1.49
Energy production and conversion						
B5EN68	2254	NADH-quinone oxidoreductase subunit D (nuoD)	49	5	2	2.95
B5EN65	2251	NADH-quinone oxidoreductase, chain G (nuoG)	45	7	6	2.48
B5EQZ1	2750	C-type Cytochrome 1 (Cyc1)	131	43	19	1.71
B5EQY9	2748	Cytochrome c oxidase subunit II (CoxB)	509	50	35	1.68
B5EQZ2	2751	C-type Cytochrome 2 (Cyc2)	302	8	18	1.57
B5EP09	850	Hydrogen: quinone oxidoreductase	125	18	12	1.52
B5EQY4	2743	Rusticyanin (Rus)	1496	56	93	1.47
B5EQY8	2747	Cytochrome-c oxidase (CoxA)	224	13	32	1.62
Cellular processes and signaling						
Post-translational modification, protein turnover, and chaperones						
B5EK73	174	Putative uncharacterized protein (CusF3)	74	18	3	6.50
B5EM36	2057	Putative uncharacterized protein (CusF2)	109	18	3	6.25
B5EPB0	2456	Band 7 protein	66	9	2	3.80
B5EQK5	2710	ResB family protein (ResB)	147	17	14	2.26
B5EQZ5	2755	Chaperone protein (HtpG)	432	28	27	1.83
B5EQK6	2711	Cytochrome c-type biogenesis protein CcsB (ResC)	90	2	1	1.40
Cell wall/membrane/envelope biogenesis						
B5EL49	1914	Phospholipase C	133	11	8	1.50
B5EQM0	1129	RfaE bifunctional protein	111	8	3	1.42
Signal transduction mechanisms						
B5EPW7	2568	Two component, sigma54, transcriptional regulator, Fis family	93	7	4	2.13
Information storage and processing						
Translation, ribosomal structure and biogenesis						
B5EM96	521	30S ribosomal protein S13 (rpsM)	129	41	12	1.44
Poorly characterized						
General function prediction only						
B5EN43	2229	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	820	55	53	1.43
Function unknown						
B5ERT0	1368	Putative uncharacterized protein	38	5	1	5.28
B5EQZ0	2749	Putative uncharacterized protein (AcoP)	299	32	19	1.55

* Score in this table refers to protein score, according to Mascot search engine.

an important role for these proteins in copper resistance in *A. ferrooxidans*. Likewise, the increased levels of $CusF3_{Af}$ exclusive of strain ATCC 53993 may contribute to the higher copper tolerance of this bacterium stressing out the importance of its GI in metal resistance as previously proposed [1].

A. ferrooxidans lives at an acid external pH (1–3) and its cytoplasmic pH is up to 5 units higher than the external pH. This generates an elevated pH gradient across the cytoplasmic membrane that contributes to the proton motive force (PMF) comprising the membrane potential ($\Delta\Psi$) and the transmembrane pH difference (ΔpH) [18]. The RND type transporters are antiporters taking advantage of the protons gradient for the efflux of copper with protons entrance to the cytoplasm. Due to its economy from the energetic point of view, these systems would be used preferentially by the cells to remove intracellular copper. A possible cytoplasmic acidification would be expected to take place if these efflux pumps were excessively used by the microorganism in the presence of high metal concentration. However, this acidification could be diminished by the energetic metabolism of the bacterium, since the oxidation of Fe(II) by molecular oxygen as the final electron acceptor consumes protons. Since the RND systems are introducing protons from the culture medium to the cell during copper detoxification an increase in the extracellular pH of the growth medium would be expected in the presence of this metal. Preliminary measurements have indicated an increase in the external pH of *A. ferrooxidans* ATCC 53993 medium under these stressing conditions (not shown). It will be of great interest to further explore this response in future studies.

Reduced levels of the main outer membrane protein Omp40 have been previously reported when *A. ferrooxidans* ATCC 23270 is grown in the presence of 40 mM copper. This was suggested as a possible adaptation to decrease entrance of toxic cations into the cell [10]. *A. ferrooxidans* ATCC 53993 on the other hand, did not decrease its Omp40 levels (Table 3). On the contrary, it showed the downexpression of a different putative outer membrane efflux protein (Lferr_1706) with

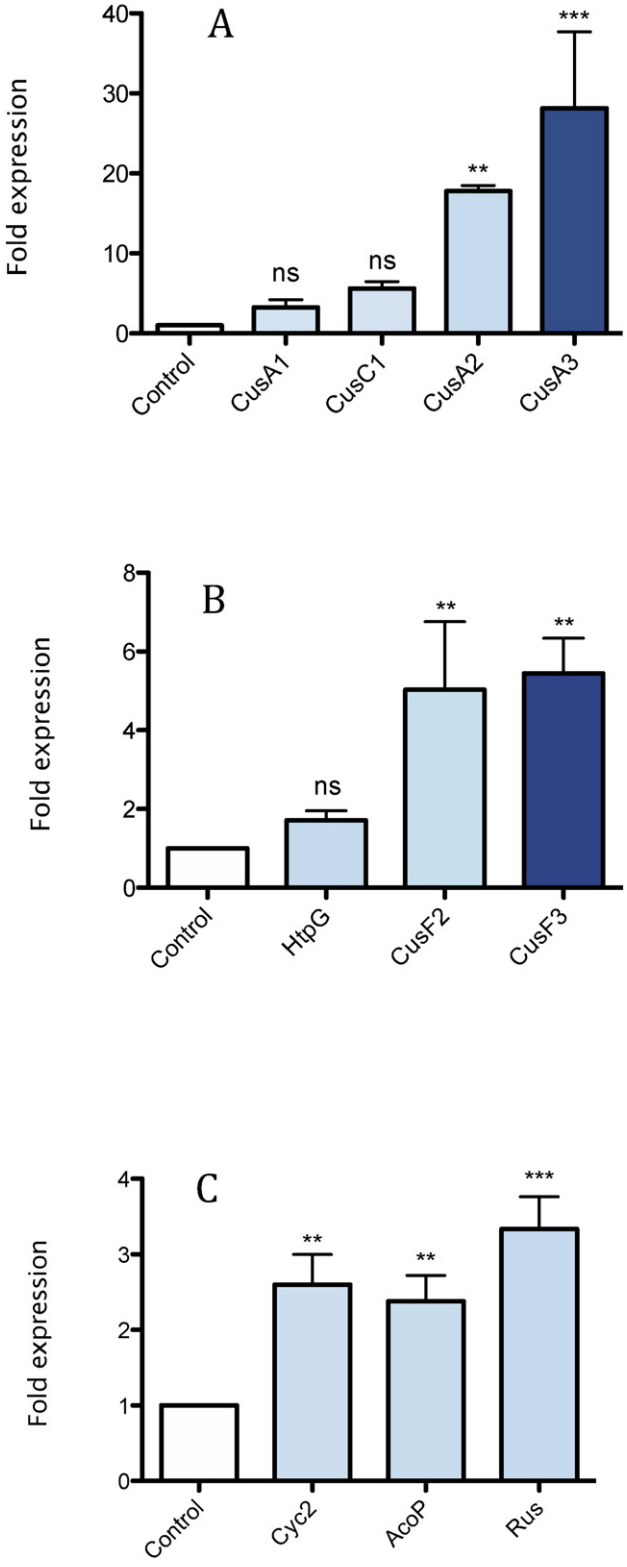


Fig. 2. Relative transcriptional expression of *A. ferrooxidans* ATCC 53993 selected genes. Genes coding for RND type efflux systems (A), different chaperones (B) and part of the *rus* operon (C) were analyzed. Cells were grown in ferrous iron and in the presence 40 mM $CuSO_4$. Transcriptional level changes refer to the control condition in the absence of copper (white bar). Genes analyzed are outside of the genomic island (light blue) or inside the GI of the microorganism (dark blue). Data were normalized by using the expression values for the 16S $rRNA_{Af}$ gene. The error bars indicate the standard deviations based on three different experimental values. Application of the one way ANOVA test and a Dunnett post-test indicated: *** $p \leq 0001$, ** $p \leq 0.01$ and * $p \leq 0.05$. ns indicates a $p > 0.05$.

similarities to TolC from *E. coli* and FusA from *Pseudomonas* (Table 2). Currently, the possible role of this change is unknown.

Iron is an essential growth factor for all bacteria since it forms part of proteins such as catalases, peroxidases, oxidases and cytochromes. It is also involved in the biosynthesis of pyrimidines, amino acids, and the carboxylic acids cycle. Due to its redox potential iron is an essential co-factor in electron transport chains [19]. Therefore, microorganisms have developed specialized systems to trap iron. One of these mechanisms involves the synthesis and liberation of siderophores with high Fe (III) affinity [19]. Once these small molecules capture iron they are recognized by TonB dependent membrane transporters and in most cases they enter the cells where they liberate the bound iron. These transporters show a high affinity and specificity for siderophores and to transport them use the proton motive force derived energy generated across the inner membrane [20].

As seen in Table 1, increased levels of a TonB-dependent receptor (Lferr_1945) were seen in *A. ferrooxidans* ATCC 53993 grown in the presence of copper. This could be due to an iron deficit generated at the cytoplasm since to respond to the stress generated by copper more iron is needed to provide the cell the iron-containing-prosthetic groups of several Heme-containing proteins such as C-type cytochromes (Cyc1 and Cyc2) and two cytochrome oxidase aa3 subunits (CoxA and CoxB). These proteins belong to the energy production and conversion subcategory. ResB and ResC (involved in the insertion of Heme prosthetic group to C-type cytochromes [21]) belong to the Cellular Processing and signaling category, sub-category posttranslational modification, protein turnover and chaperones. All these proteins increased their levels in *A. ferrooxidans* ATCC 53993 as seen in Table 1. Considering that cytochromes are essential for the energetic metabolism of this acidophile the overexpression of these and other metabolic components in the presence of copper would be expected due to a higher energy demand required to handle the stress generated by the metal. Furthermore, the overexpression of these porphyrin-containing molecules might play an important role in iron homeostasis [22].

3.2. Metabolism/carbohydrates and amino acids transport and metabolism

A possible hexokinase (Lferr_2462) showed increased levels of expression in the presence of copper (see Carbohydrate transport and metabolism subcategory in Table 1). This enzyme catalyzes the reaction forming glucose-6-phosphate from glucose. This phosphorylated sugar can enter the glycolytic and the pentose phosphate pathways. On the other hand, phosphofructokinase-1 that converts fructose-6-phosphate to fructose-1.6-biphosphate was not identified. In addition, the levels of fructose-biphosphate–aldolase (Lferr_1374) that catalyzes the formation of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate from fructose-1.6-biphosphate were decreased (Table 2). These results suggest that the glycolytic pathway is not preferred in the presence of copper. Furthermore, the lower levels of a possible transketolase (Lferr_1364), which would connect the pentose pathway with the glycolytic pathway, and the decreased levels of ribulose diphosphate carboxylase (Lferr_2661) (Table 2) which converts ribulose-1.5 diphosphate to 3-phospho-glycerate suggests the pentose phosphate pathway is favored over the glycolytic pathway in cells subjected to copper. This would provide higher reducing power in the form of NADPH and ribose to respond to the stressing condition.

Regarding the Amino acid transport and metabolism subcategory, glutamate synthase increased its levels (Lferr_0878 in Table 1). This enzyme synthesizes glutamate from glutamine and 2-oxoglutarate. Glutamate can be used by glutamate cysteine ligase to generate γ-glutamylcysteine which in turn is converted to glutathione by glutathione synthase. Glutathione is known to protect cells from reactive oxygen species (ROS) and free radicals and peroxides. The transcriptional overexpression of some of these enzymes has been previously reported in *A. ferrooxidans* ATCC 23270 grown in copper-containing ores [6]. Also increased levels of glutamate cysteine ligase were previously reported

Table 2
Down-Regulation of proteins in *A. ferrooxidans* ATCC 53993 grown in ferrous iron and in the presence of 40 mM $CuSO_4$.

Accession number	ORF	Function/similarity	Score*	Coverage (%)	Peptide number	Fold change (Cu 40/0 mM)
Metabolism						
Inorganic ion transport and metabolism						
B5EJZ7	1706	Outer membrane efflux protein	49	5	2	0.25
B5EQV4	1216	Extracellular solute-binding protein family 1	37	7	2	0.33
B5EMQ2	2188	Rhodanese domain protein	95	18	1	0.58
Carbohydrate transport and metabolism						
B5ERT6	1374	Ketose-bisphosphate aldolase class-II	217	16	6	0.60
B5ERS6	1364	Transketolase central region	691	22	46	0.60
Lipid transport and metabolism						
B5EQ16	2617	Beta-ketoacylsynthase	70	2	7	0.45
Energy production and conversion						
B5EQF8	2661	Ribulose-bisphosphate carboxylase	127	22	5	0.62
B5ES47	1490	Pyruvate dehydrogenase (Acetyl-transferring)	123	15	6	0.60
B5ERS1	1359	NADH/Ubiquinone/plastoquinone (Complex I)	204	9	6	0.59
Cellular processes and signaling						
Cell wall/membrane/envelope biogenesis						
B5EKC7	1740	Glycosyl transferase group 1	58	5	3	0.45
B5EJS6	1635	ADP-L-glycero-D-manno-heptose-6-epimerase	39	2	1	0.41
Poorly characterized						
General function prediction only						
B5ES48	1491	Alkyl hydroperoxidase like protein, AhpD family	128	22	4	0.56
Function unknown						
B5EL34	1899	Pyrrolo-quinoline quinone	51	8	6	0.14
B5EM48	2073	Putative uncharacterized protein	50	8	1	0.37
B5ELW0	478	Putative uncharacterized protein	119	53	11	0.62
B5ERS0	1358	UPF0753	966	33	63	0.63

* Score in this table refers to protein score, according to Mascot search engine.

in strain ATCC 23270 grown in the presence of copper [10]. On the contrary, this protein showed no changes in strain ATCC 53993 in the presence of this metal. As already mentioned, this last strain is much more resistant to the metal than the former one [1]. It can be speculated then that the effects of copper in generating oxidative stress are much lower in strain ATCC 53993 in the presence of 40 mM copper.

On the other hand, glutamate can be used to form Glu-tRNA by Glu-tRNA synthetase (GluRS). Glu-tRNA is not only used for the biosynthesis of proteins but also in the formation of tetrapyrroles using the C5 pathway. It has been reported that *A. ferrooxidans* generates an extremely high number of the respiratory chain components per cell [23]. The level of these components including cytochromes, and therefore Heme groups, depends on the growth conditions and Glu-tRNA [24]. Considering that several cytochromes increased their levels in the presence of copper (Table 1) it can be suggested that heme formation is favored in the presence of the copper concentration tested, and this could be related with a higher respiratory requirement under toxic conditions.

A. ferrooxidans ATCC 23270 subjected to 40 mM copper showed increased levels of several proteins involved in the biosynthesis of histidine and cysteine [10]. This may be due to the participation of these amino acids in copper binding sites of proteins such as metal chaperones. On the contrary, this kind of changes was not seen in strain ATCC 53993 at the same copper concentration (Table 3). To determine if this response is a more general phenomenon, it will be interesting to determine whether similar changes related to the biosynthesis of His and Cys also take place in *A. ferrooxidans* ATCC 53993 grown at higher copper concentrations.

3.3. Energy production and conversion

Electrons obtained from Fe (II) oxidation by *A. ferrooxidans* can have two different pathways [25]. One of these routes involves the proteins coded in the *rus* operon: an outer membrane cytochrome c (Cyc2) (Lferr_2751), a periplasmic protein recently named AcoP (Lferr_2749) [26], a blue copper periplasmic protein Rus (Lferr_2743), a periplasmic c class I cytochrome (Cyc1) and a cytochrome oxidase aa3 type (coxBACD) (Lferr_2747/2750) [27]. The second path includes a bc1 cytochrome complex (complex III, ubiquinol-cytochrome c reductase), a quinones pool and an NADH dehydrogenase I complex. The genes coding for bc1 complex are forming part of the *petI* operon [28].

Table 3
Comparison of the expression levels of some selected proteins in *A. ferrooxidans* strains ATCC 23270 versus ATCC 53993[a].

Protein	ATCC 23270 (ORF)	Fold change	ATCC 53993 (ORF)	Fold change
CusA1	Afe_1947	↑↑↑	Lferr_1617	–
CusB1	Afe_1948	↑↑↑	Lferr_1618	↑↑
CusC1	Afe_1949	↑↑↑	Lferr_1619	↑↑
CopZ	Afe_1862	↑↑↑	Lferr_1538	–
Cation channel	Afe_3093	↑↑	Lferr_2691	–
Omp40	Afe_2741	↓	Lferr_2362	–
OmpA	Afe_2685	↓	Lferr_2309	–
TonB receptor	Afe_2040	↓	Lferr_1702	↑
Cyc2	Afe_3153	↓	Lferr_2751	↑
Cyc1	Afe_3152	↓	Lferr_2750	↑
Rus	Afe_3146	–	Lferr_2743	↑
CydA	Afe_0955	↑↑↑	Lferr_1070	–
CydB	Afe_0954	↑↑↑	Lferr_1069	–
PDH	Afe_3068	↑↑	Lferr_2674	–
Cbb1	Afe_3051/3052	↑↑↑	Lferr_2660/2661	↓
Cbb2	Afe_1690/1691	↓	Lferr_1388/1389	–
His operon	Afe_3040-3046	↑↑[b]	Lferr_2649-2655	–
Cys operon	Afe_3121-Afe_3125	↑↑[c]	Lferr_2723-2727	–
Glutaredoxin	Afe_3038	↑↑	Lferr_2647	–
Glutamate Cys ligase	Afe_3064	↑↑	Lferr_2670	
Glutathione synthetase	Afe_3064	↑↑	Lferr_2671	–
DsbG	Afe_1943	↑↑↑	Lferr_1615	–
RND copper	Afe_1878-1880	–	Lferr_1553-1555	–
UbiH-2	Afe_2388	↑↑↑	Lferr_2018	–
MurA	Afe_3039	↑↑	Lferr_2648	–
AhpD	Afe_1814	–	Lferr_1491	↓

↑: 1–3 fold increase; ↑↑: 3–6 fold increase; ↑↑↑: more tan 6 fold increase. ↓: <1 fold decrease; (−): not significant value.

[a] Both strains were grown in the presence of ferrous iron and 40 mM $CuSO_4$ and compared with their respective controls in the absence of the metal. Data from ATCC 23270 were taken from Almarcegui et al., 2014.

[b] Seven of the eight genes in the His operon.

[c] Five genes related to Cys operon.

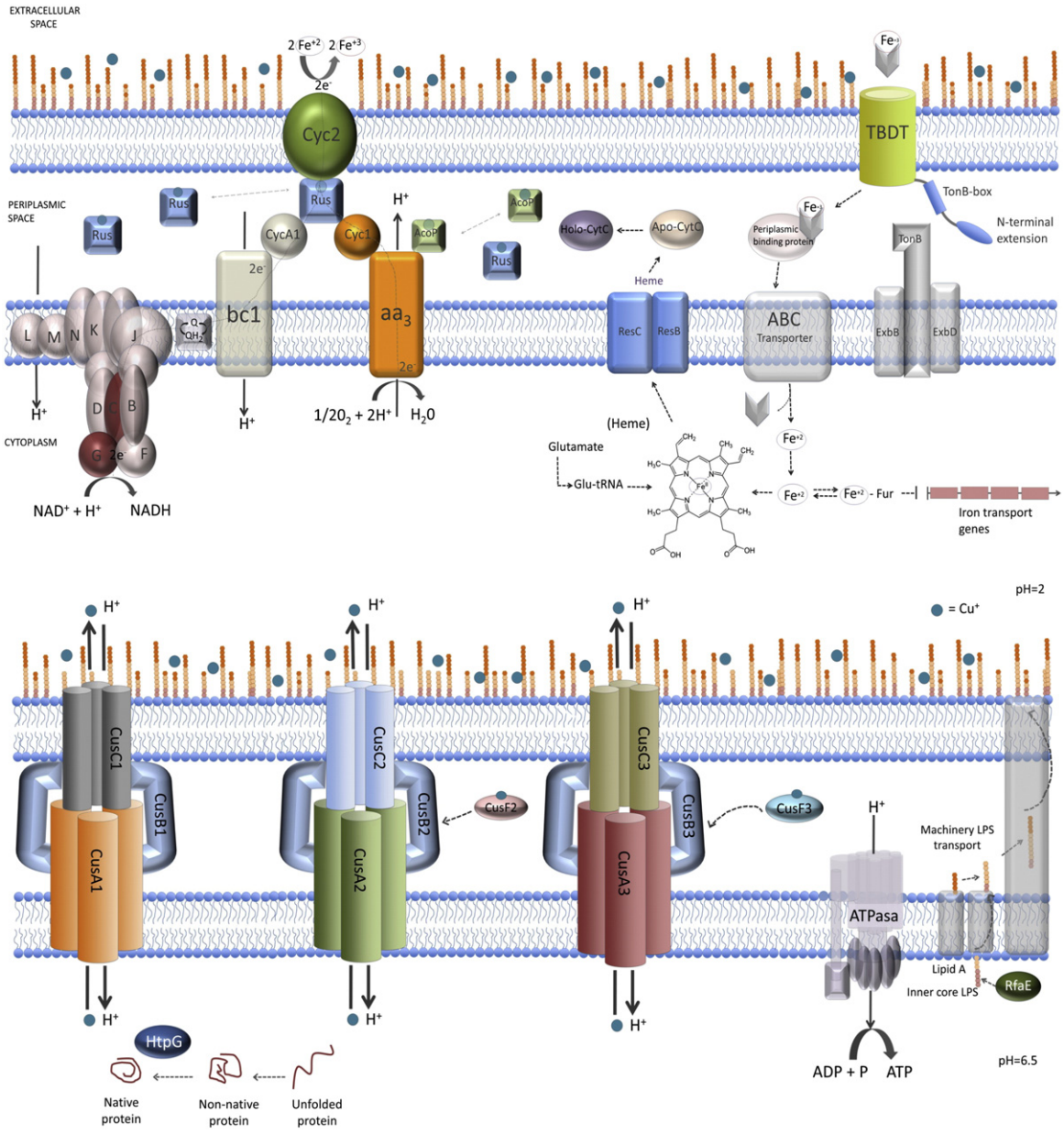


Fig. 3. Working scheme showing most of the proteins with increased levels in *A. ferrooxidans* ATCC 53993 exposed to copper. Proteins in different colors are those that increased their levels in the presence of the metal. Those in white color are some cellular components that did not change and are included only to illustrate their possible interactions with the experimentally determined proteins. The order and locations of the predicted proteins is arbitrary.

It has been proposed that Cyc2 located at the inner membrane accepts the electrons directly from Fe (II), being the first step in iron oxidation [29]. These electrons are then directed to Rus. At this point, part of the electrons can go to through Cyc1 towards cytochrome oxidase aa3 which finally reduces oxygen to water. Alternatively, electrons can go towards CycA1 which also receives electrons from Rus and are delivered onto the bc1 complex and ubiquinones to be finally supplied to NADH dehydrogenase I to generate reducing power [28] (see Fig. 3). Table 1 shows an increased level of almost all proteins codified by the *rus* operon at 40 mM Cu. As previously reported, *A. ferroxidans* 53993 growth was not affected by the presence of this copper concentration [1]. Therefore, it is possible that under these metal concentrations respiration would not be affected in this strain. On the other hand, it has been recently proposed that in addition to its energetic role Rus and AcoP could be important copper resistance determinants since they not only could bind excess copper in the periplasm but are also overexpressed in *A. ferrooxidans* grown in Fe(II) or elemental sulfur in the presence of copper [30].

3.4. Cellular processes and signaling

The alternative transcriptional sigma factor 54 (σ54) is crucial to activate the response of the cell to a number of external signals. σ54-dependent genetic expression regulates specific and fast responses to environmental changes [31]. Among these responses are bacterial virulence, flagellar synthesis, adherence and biofilm formation [32,33]. On the other hand, it has been reported that σ32 and σ54 regulate the expression of the HtpG molecular chaperone from *E. coli* (homologous to Hsp90) which is overexpressed under heat shock [34,35], acid stress [36] and nitrogen starvation [37].

Table 1 shows increased levels of a putative two component σ54 transcriptional regulator of the Fis family (Lferr_2568). When

Lferr_2568 is activated, it allows a conformational change of σ54 to start transcription of genes mediated by this factor [38]. In *A. ferrooxidans*, this putative transcriptional regulator could activate the expression of specific stress genes regulated by σ54 such as chaperone HtpG (Lferr_2755) (see below) to respond to the shock and damage generated by copper.

3.5. Cell wall/membrane/envelope biogenesis

Gram-negative bacteria possess a cell envelope that in addition to the peptidoglycan contains an extra layer composed of lipopolysaccharide. This is a second lipidic layer containing not only phospholipids but also polysaccharides and proteins. The capacity of Gram-positive bacteria to bind metals is mainly due to the peptidoglycan layer [39]. On the contrary, in Gram-negative microorganisms this layer is thinner and therefore it is less likely that these bacteria have the same metal binding capacity than Gram positive ones. Since the lipopolysaccharide is a highly anionic structure with capacity to bind metallic cations without spending cellular energy [40] it has been proposed to be the main metals binding site in Gram-negative cells [41].

Table 1 shows that in *A. ferrooxidans* subjected to copper the levels of RfaE bifunctional protein (Lferr_1129), which is involved in lipopolysaccharide synthesis were increased. Specifically, this protein is involved in the synthesis of D-glycero-D-mano-heptose-1-phosphate and in the transfer of ADP to generate ADP-D-glycero-D-mano-heptose, a precursor of the internal nucleus of LPS [42]. This suggests that LPS might be the first important barrier to protect this acidophile from metal stress. Obviously, further experimental work will be required to support this idea.

3.6. Post-translational modification, protein turnover and chaperones

The Hsp90 (HtpG en *E. coli*) family of chaperones constitutes up to 1–2% of the total cytosolic proteins in eukaryotes and their abundance can increase about 2-fold under stress conditions [43]. These chaperones interact specifically in the late stages of folding of a group of proteins involved in regulatory and signaling pathways such as transcription factors and protein kinases [44]. Hsp90 chaperones are also involved in the reactivation of denatured or inactivated proteins during environmental stress in prokaryotes and eukaryotes [45,46]. Thus, Hsp90 synthesis is promoted by all kinds of stress, such as oxidative stress, damage by free radicals, exposure to heavy metals and spontaneous mutations [47,48]. On the other hand, it is known that the levels of these heat shock proteins are regulated by σ54 [49].

Table 1 shows increased levels of an *A. ferrooxidans* ATCC 53993 chaperone (Lfer_2755, annotated as HtpG) when the microorganism was grown in the presence of copper. This result was confirmed by determining the transcriptional levels of its gene (Fig. 2B). These findings suggest that this protein could be involved in the correct folding and repair mechanisms of proteins damaged by the presence of copper during the growth of the microorganism.

3.7. Poorly characterized/general function prediction only

Table 2 showed a marked decrease level of an alkyl hydroperoxidase (AhpD) (Lferr_1491) by the presence of copper. AhpC and AhpD are components of alkyl hydroperoxide reductase that participates in defense against ROS. It has been determined that AhpD acts as a thioredoxin to reduce the cysteine residues of AhpC and thus complete its catalytic cycle [50,51]. The alkylhydroperoxide species are able to initiate and propagate a free radicals chain reaction that results in DNA and membranes damage. The enzyme AhpC can destroy toxic hydroperoxide intermediates and repair peroxidized molecules. Therefore, it has an important role in the detoxification derived from oxidative stress [52]. Furthermore, the levels of expression of these enzymes may represent a measure of the oxidative stress present in the cell.

Considering that very few oxidative stress-related enzymes changed their expression levels in *A. ferrooxidans* ATCC 53993 in the presence of 40 mM copper it is possible that the oxidative stress generated in this strain grown in the presence of the metal is much lower than that of strain ATCC 23270 in which several proteins related to oxidative stress (including proteins from the AhpC family) were upregulated under the same conditions [10].

It was previously reported that several proteins related to oxidative stress, such as glutaredoxin (Afe_3038), glutathione synthetase (Afe_3063) and Ubih-2 (Afe_2388) involved in ubiquinone synthesis were overexpressed in *A. ferrooxidans* ATCC 23270 exposed to 40 mM Cu^{2+} [10]. As seen in Table 3, on the contrary, when *A. ferrooxidans* ATCC 53993 was exposed to the same copper concentration the oxidative stress related proteins were not detected by the same ICPL proteomic procedure.

DsbG-L, a protein most likely involved in the repair of non-native disulfide bonds generated by metal stress at the periplasmic space [53] had highly increased levels in *A. ferrooxidans* ATCC 23270 subjected to copper [10]. On the other hand, this protein although present in its genome, was not detected in ATCC 53993 cells exposed to copper as seen in Table 3. Finally, several proteins (Afe_1878-Afe_1880), possibly forming an RND system involved in copper efflux were overexpressed in strain ATCC 23270 as detected by using 2D PAGE [10]. Although these proteins were also detected by the ICPL analysis in ATCC 53993 grown in the presence of 40 mM copper, they did not show a significant change in their levels (Table 3).

Strain ATCC 53993 changed the levels of several proteins not previously seen in ATCC 23270 under the presence of 40 mM Cu (II) (Tables 1 and 2) suggesting that the microorganism is already being affected by the metal and somehow is initiating a response to the toxic metal. As seen in Table 3, both strains of *A. ferroxidans* showed very high levels of the components of their Cus systems, supporting their importance in copper resistance.

Fig. 3 shows a working model where most of the proteins studied here increasing their levels in the presence of copper are summarized. In conclusion, the proteomic approach is very useful to analyze the global response to metals of microorganisms such as *A. ferrooxidans* where very few genetic tools are available. The results presented here strongly support the importance of the expression of genes in the exclusive GI of strain ATCC 53993 in its resistance to copper. Compared with strain ATCC 23270, the former one is able to outgrow the last one when grown together at high copper concentrations. This knowledge is important not only to understand better copper resistance mechanisms in acidophiles but also to select more efficient microorganisms for biomining operations.

Conflict of interest

The authors declare to have no conflict of interest.

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